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AFRICAN SWINE FEVER VIRUS: USE OF GENETIC MARKERS IN ANALYSIS OF ITS ROUTES OF SPREAD

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SUMMARY

At present no effective measures for specific prevention and treatment of African swine fever have been developed. The control strategy for the disease is designed for rapid diagnosis of infected animals with subsequent slaughter and decontamination (stamping out). The present review deals with current epidemic situation for African swine fever and examines features of the virus genomics and genetic differentiation of the isolates. The Russian Federation has been ASF-infected since 2007. Since that time the disease has been one of the key problems in pig farming of this country inflicting great economic losses, both directly and indirectly. The disease continues to spread. In January 2014 African swine fever was introduced to Lithuania, then pervaded Poland, Latvia, Estonia, Romania, Belgium and Moldova. Since 2018 the disease outbreaks have been reported in Asia (China, Vietnam, and Mongolia). Specific structure of the virus and long genome, encoding genes with unknown function, and circulation of 24 genotypes and 9 serotypes of the virus hinder the development of ASF vaccine. The article shows that the use of many specific genetic markers during determination of relationship and study of pathways of ASF virus global spread is the most accurate method.

Key words: African swine fever, structure of virus genome, genetic markers.

CURRENT SITUATION

African swine fever (ASF) is a highly dangerous hemorrhagic viral disease. It is an OIE-listed disease that must be reported due to high mortality rate among infected animals, wide spread and strong sanitary and social and economic impact on pig farming as an industry and on global trade in its products [8].

The disease is caused by the only member of the *Asfarviridae* family – a double-stranded DNA virus.

ASF epidemic is complex and varies significantly in dynamics and factors of spread between countries, regions and continents showing diverse development scenarios.

Until recently ASF was endemic in countries of sub-Saharan Africa [5], and Europe with outbreaks in Portugal, Spain (1957–1995) and Sardinia (since 1978). Taking into account the present situation in Europe, it is important to note that after the introduction of ASF virus in Georgia from East Africa in 2007, the disease rapidly spread to all Caucasus and thousands of kilometers further to the north-west of the Russian Federation [2, 7]. It also extended westwards – to Europe (2014–2019) and South-East Asia (2018–2019).

Clinical signs of ASF observed in infected animals in 2013–2015 in the EU countries were typical for the acute disease [13, 16]. However, a year after ASF occurrence in Europe researchers detected seropositive wild boars, which suggests that there have been recovered animals. This was confirmed by *in vivo* tests [9, 17, 27].

Seriousness of ASF spread is attested by outbreaks in Moldova, Czech Republic and Romania in 2017, in Hungary, China, Belgium and Mongolia in 2018, in Vietnam, Cambodia, Democratic People's Republic of Korea, Lao People's Democratic Republic and in the Far Eastern region of the Russian Federation (Fig. 1) [3, 35].

Thus, currently ASF constitutes a real threat for pig farming not only due to expanding distribution of the already circulating virus but also due to its possible re-introduction from endemic African countries the number of which is ever growing.

Primary introduction of ASF agent to the population of domestic pigs or wild boars results in peracute, acute or subacute disease with mortality rate as high as 95–100% (in 4–9 days after infection).

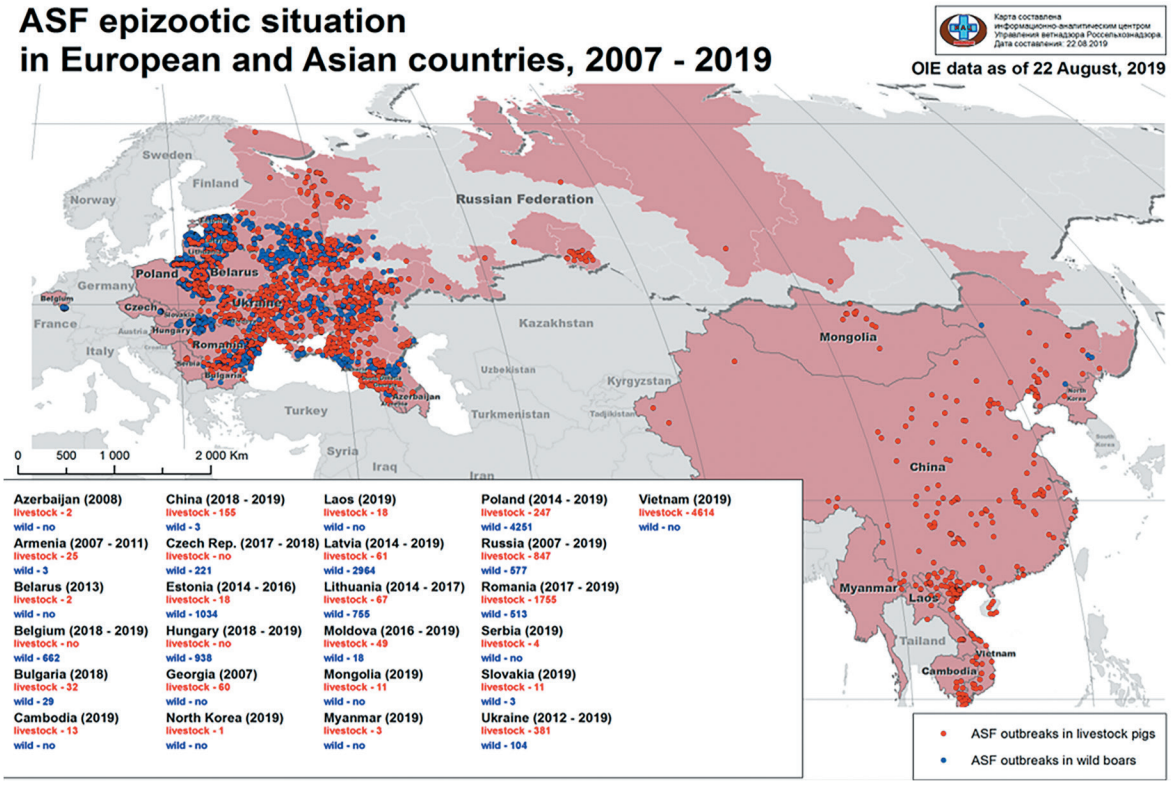


Fig. 1. ASF epizootic situation in Russia, European and Asian countries in 2007–2019 [3]

However, longtime circulation of ASF virus in the wild nature creates attenuated virus variants which cause chronic or even subclinical course of the infection. When the disease is caused by a low-virulent virus, ASF clinical manifestations are more diverse or not readily detected. ASF agent can reside in the organism of infected animals for several months without producing evident clinical signs [10, 12].

Under certain conditions an active role in the ASF spread is played by transmission vectors. Thus, for example, for centuries ASF virus circulation in East and South Africa is sustained in sylvatic cycle comprised of soft ticks of *Ornithodoros* genus and subclinically infected wild red river hogs (*Potamochoerus porcus*) and warthogs (*Phacochoerus* spp). The sylvatic cycle of ASF virus that has formed between ticks and wild boars can be maintained indefinitely which ensures virus circulation and its survival in the wild while keeping the initial virus and generating new variants.

There is no vaccine for ASF prevention. Control and eradication measures in place are based on traditional methods to combat diseases, including epidemiological surveillance, epidemiological investigation, rapid diagnosis of infected animals and stamping out policy [11].

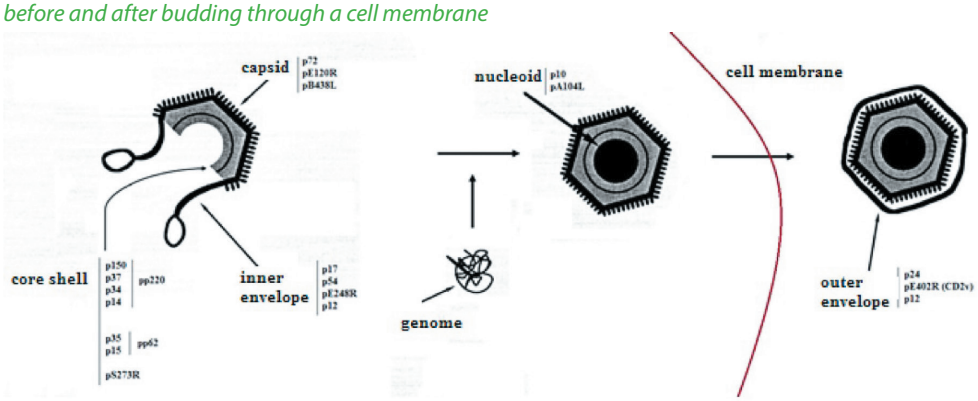
Longtime circulation of ASF virus in the wild is attributed both to its unique biological properties and its long-term survival in the outside environment which is underpinned by a complex structure of the virion.

STRUCTURE OF ASF VIRUS

ASF virus is icosahedral in morphology, about 200 nm in diameter and consists of a nucleus made of a central nucleoid with genome and surrounded by several concentric layers: a dense nucleoprotein layer, inner lipid membrane and a capsid.

Extracellular virions have an additional external envelope derived by budding from cell membrane [14].

Fig. 2. Structural components of the virion and localization of proteins before and after budding through a cell membrane



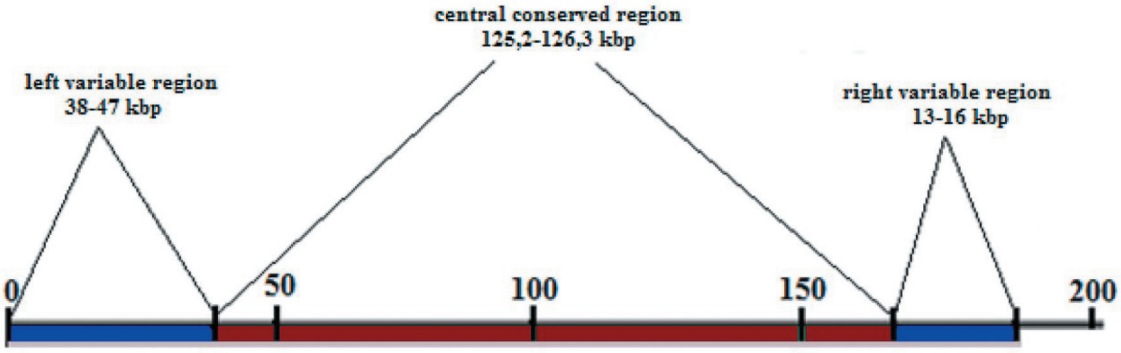


Fig. 3. Map of ASF virus genome

Two-dimensional analysis of purified extracellular virus revealed 54 structural proteins with molecular weight from 10 to 150 kDa.

Currently, 19 genes of structural proteins are known [8], localization of which is determined in different layers of the virus particle (Fig. 2).

As it can be seen in Figure 2, the virion of ASF virus has an envelope made of 5 protein groups. Each group comprises two and more structural proteins.

In total, more than 160 viral proteins are synthesized in ASF virus replication and they have different functions varying from participation in virus replication to regulation of intracellular synthesis [8]. With that, functions of nearly half of proteins of ASF virus are yet to be studied in detail.

Similar to other complex DNA viruses, ASF agent has proteins responsible for a number of processes involved in evading host defence systems, including innate and acquired immune mechanisms such as suppression of type I interferon production, regulation of apoptosis, development of hyperergic inflammation and activation of expression of cell host immunomodulatory genes [4, 6].

FEATURES OF ASF VIRUS GENETIC STRUCTURE

ASF virus is commonly acknowledged to have come from East Africa. The disease was firstly described in 1921 in Kenya, after the first outbreak in 1903. Nonetheless, TMRCA estimates (TMRCA – time to the most recent common ancestor) determined that the most recent ancestor of all ASF virus isolates circulating now appeared about three centuries ago, i.e. in the early XVIII century.

The genome of ASF virus comprises from 150 to 167 open reading frames (ORF), its size, depending on the isolate, varies from 170 to 190 kb (kbp). The virus genome has a conserved central region and right and left variable regions.

Initially, variants of ASF virus were differentiated on the basis of restriction analysis and genome size. High variability was mostly observed within the range of 38–47 kbp on the left end and 13–16 kbp on the right end of the genome (Fig. 3). Those two variable regions comprise multigene families (MGF) which play a significant role in regulation of gene expression and differ in number of tandem repetitions.

Besides, the variety of ASF virus variants is due to a variable number of amino acid repeats in 14 proteins, including central variable region (CVR) encoded by B602L gene and membrane protein p54 encoded by E183L gene [15, 26, 30].

Compared to other large DNA viruses such as gammaherpesvirus of vertebrates (10^{-9} substitutes/position/year) and small DNA viruses, for example, Cunningham virus (10^{-7} substitutes/position/year), ASF virus is characterized with a high evolutionary rate of variability ($3{,}31 \times 10^{-4}$ substitutes/position/year with exponential growth of 0,01 per year^{-1}).

Analysis of nucleotide sequences of ASF virus genome established that its substitution rate varies from 10^{-4} to 10^{-5} which is more common for RNA viruses which generally have evolution rate from 10^{-2} to 10^{-5} substitutes/position/year [31].

Passaging and/or growth adaptation of ASF virus in continuous cell cultures are also characterized with a high variability rate of its genome, the analysis of which demonstrates multiple substitutions and insertions as well as appearance of large deletions (up to 3000 bp) both in right and left variable regions [1].

Selection cloning was used to prove that natural populations of ASF virus were represented with various genetic and antigenic variants. Clonal analysis of ASF virus isolates showed that their populations can be heterogeneous by composition, therefore, according to R. Blasco et al. (1989), any classification of virus isolates should proceed from data on conserved central region of DNA molecule [34].

PRINCIPLES OF GENETIC DIFFERENTIATION OF ASF VIRUS ISOLATES

Due to complicated classification of ASF virus isolates from East and South Africa, the system of genetic grouping was based on the sequence analysis of highly conserved B646L gene encoding major viral protein vp72 [26]. Phylogenetic analysis with Bayesian estimation and nearest neighbours method established that natural evolution of ASF virus can be traced down by B646L gene which reportedly has no multiple recombination, non-synonymous substitutions or codons with positive natural selection.

Initially available and studied isolates were classified into 10 genotypes.

When West African, European and South American isolates were proved to be highly homologous, they were grouped into genotype I. This contributed to identifying possible introduction ways of the virus of the given genotype to different continents.

Further phylogenetic analysis of ASF outbreaks demonstrated that genotype I was ever spreading in West and Central Africa [22], and in countries such as Uganda and Kenya the virus of genotypes IX and X was permanently present.

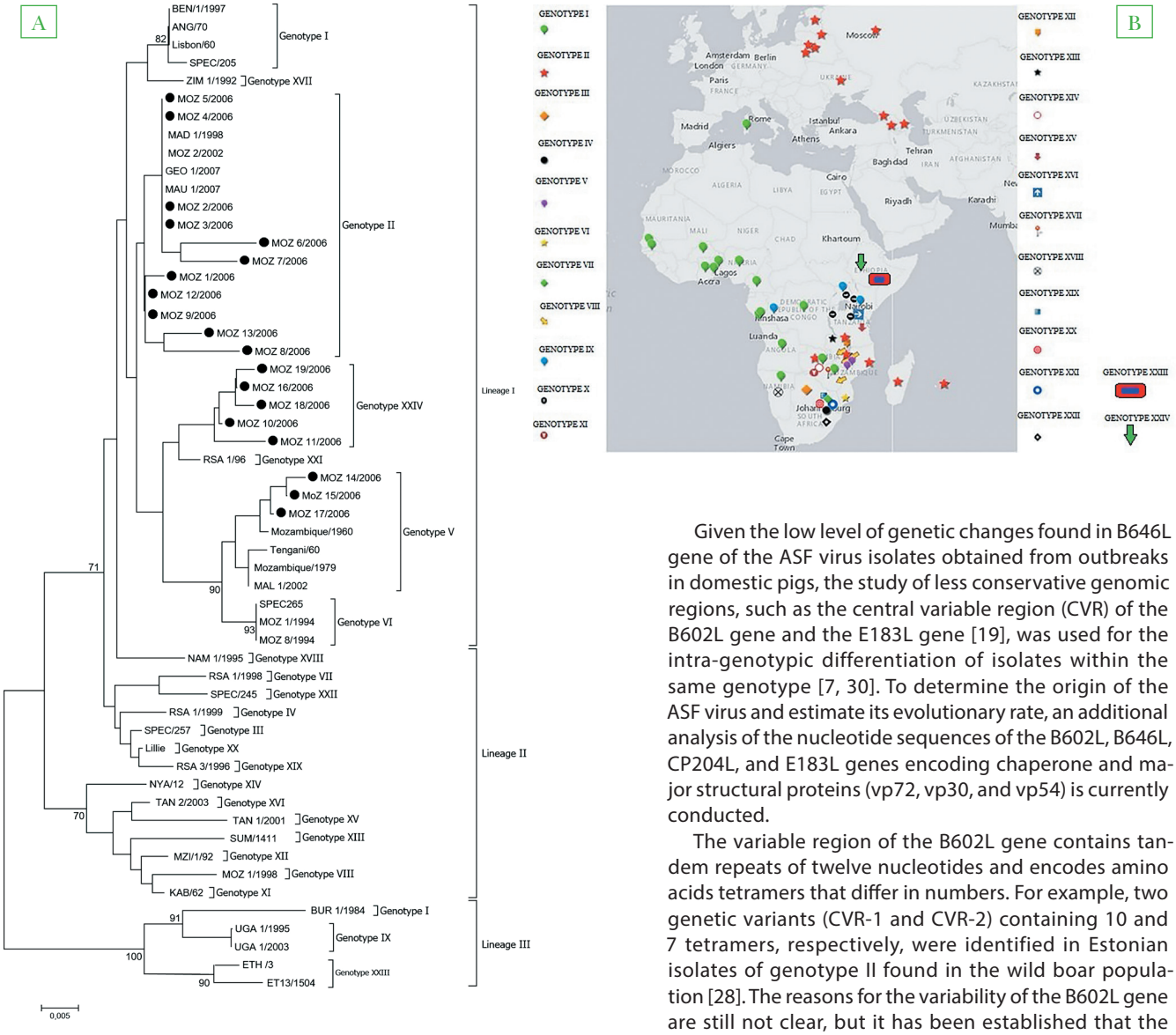


Fig. 4. ASF virus genotypes

A – phylogenetic analysis based on the B646L gene sequence of 55 ASF virus isolates representing 24 genotypes;
B – spread of ASF virus genotypes in the world.

Isolates from East and South Africa are divided into 21 genotypes [26, 29]. The fact suggests that as a rule the variety of ASF virus variants is generated in the sylvatic cycle [18]. By now, genotype XXIV of ASF virus which was isolated from soft ticks has been identified in Mozambique [20] (Fig. 4).

The ASF virus genotype II is still present in Tanzania, Mozambique, Madagascar and Zambia, and as a result its spread led to epidemic in Georgia in 2007 [21]. Since that time, ASF has spread from the Caucasus to the Russian Federation, Ukraine, the Baltic countries (Estonia, Latvia and Lithuania) and Poland, then to China and other Asian countries [23].

In recent years full-length genome sequences of virus strains of various genotypes have been determined [15, 25, 32], that allows not only identifying some genomic markers that can be used for genotypic differentiation of isolates, but also variable regions that make it possible to carry out intra-genotypic differentiation of closely related ASF virus isolates.

Given the low level of genetic changes found in B646L gene of the ASF virus isolates obtained from outbreaks in domestic pigs, the study of less conservative genomic regions, such as the central variable region (CVR) of the B602L gene and the E183L gene [19], was used for the intra-genotypic differentiation of isolates within the same genotype [7, 30]. To determine the origin of the ASF virus and estimate its evolutionary rate, an additional analysis of the nucleotide sequences of the B602L, B646L, CP204L, and E183L genes encoding chaperone and major structural proteins (vp72, vp30, and vp54) is currently conducted.

The variable region of the B602L gene contains tandem repeats of twelve nucleotides and encodes amino acids tetramers that differ in numbers. For example, two genetic variants (CVR-1 and CVR-2) containing 10 and 7 tetramers, respectively, were identified in Estonian isolates of genotype II found in the wild boar population [28]. The reasons for the variability of the B602L gene are still not clear, but it has been established that the vrB602L protein is a chaperone that is actively involved in capsid assembly, although it is not incorporated into virions [30].

The gene encoding the structural protein p54 is driven by positive selection pressure of the immune system, which modulates the evolution of the E183L gene and leads to the appearance of strong non-synonymous substitutions and recombinations in its sequence [19].

However, analysis of the three B602L, B646L, and E183L genes is not enough for the differentiation of isolates from one country or one region. Additional markers in the ASF virus genome are needed for grouping closely related isolates, as well as determining pathways of the virus distribution, and studying the stability of the viral genome and its rate of variability.

An improvement in the phylogenetic resolution between closely related isolates can be achieved using analysis of other viral genes, such as KP86R, I196L, and intergenic regions I73R/I329R, I78R/I215L, MGF-505 9R/10R [19, 30].

A comparative analysis of the ASF virus genome variable regions containing an array of tandem repeats allowed us to obtain more information about the origin of the isolates and to divide them into subgroups, even if they were grouped together based on the study of sequences of more conservative regions [30].

The insertion in the intergenic region I73R/I329L was first discovered by C. Gallardo et al. during the determination of the main loci of variability of the ASF virus genome. For this purpose, the authors used primers flanking this region and obtained 356 bp genome sequences of isolates from Lithuania (LT14/1482, LT14/1490) and Poland (Pol14 / Sz and Pol14/Krus). A comparative analysis of these sequences with nucleotide sequences of previously isolated strains from the Russian Federation was also carried out. The studies revealed a direct tandem repeat (TRS) of 10 nucleotides (GGAATATATA) in isolates LT14/1482, LT14/1490, Pol14/Sz and Pol14/Krus [23].

Genome-wide sequencing of Russian ASF virus isolates isolated in 2013–2014 in the ASF reference laboratory of FGBI "ARRIAH" showed that Odintsovo 02/14 isolate had a direct tandem repeat (GGAATATATA) of 10 nucleotides (IGR-2) located in the right terminal region of the genome in the intergenic region between the genes I73R and I329L.

Later, Russian specialists first discovered the 17-nucleotide TRS insertion in the intergenic region MGF-505 9R/10R-(MGF-2) in Kashino 04/13, Karamzino 02/13 and Shihobalovo 10/13 isolates. Subsequently, using primers flanking this intergenic region, the authors studied more than 40 Russian ASF virus isolates (from 2007 to 2016), and only 7 isolates from the Tver, Vladimir, and Smolensk Oblasts showed a similar insertion [33].

According to the results of these studies, Russian isolates are divided into three groups: isolates containing the tandem repeat in MGF 9R/10R (MGF-2), containing the tandem repeat in I73R/I329L (IGR-2) and not containing IGR-2 or MGF-2, i.e., have IGR-1 or MGF-1.

In 2017 TRS was detected in the intergenic region MGF-505 9R/10R, in the genome of 9 Polish isolates. The genome of these isolates contained two tandem repeats simultaneously: one – in I73R/I329L (IGR-2) and the other – in MGF 9R/10R (MGF-2). All Polish ASF virus isolates under study, except for one, Pol17/WB-CASE237, contained TRS in I73R/I329L. In addition, it was found that the genome of one Russian isolate Tver1112/Zavi contains two identical insertions in MGF 9R/10R (MGF-3), that was not observed in other isolates [C. Gallardo, 2018, unpublished data].

The results of numerous studies have confirmed that the analysis of a large number of genetic markers in determining relationship and studying the pathways of the ASF virus gives more accurate information. So, for example, in the case of determining the ways of ASF virus introduction into China, when analyzing Chinese ASF virus isolates by sequencing and comparison with other isolates from the Russian Federation and Eastern Europe, only marker regions of TRS in I73R/I329L, C-terminus of the B646L, CP204L и E148L genes were initially used. As a result of the first stage of the analysis, the researchers were able to establish only the presence of TRS in the intergenic region I73R/I329L. However, with an increase in the number of these markers taken for analysis and including the gene sequences C84L, MGF_360-1L, I267L, DP60R and MGF_360-16R, it was found that the ASF virus isolates Pig/HLJ/2018 and DB/LN/2018 were most similar to isolates isolated in Poland (PoL/2017) [24].

CONCLUSION

African swine fever is arguably the most dangerous porcine disease in the world. The disease causes large economic losses in industrial and private pig farming.

One of the most important control stages of this disease is monitoring and studying the pathways of the ASF virus. Identification of specific genetic markers remains the most accurate method for the virus grouping and studying its spread.

In 2019 the Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor) reported that in 2007–2019 more than 1,400 ASF outbreaks were recorded, which resulted in death of about 2 million pigs in 48 regions of Russia [33].

Current ASF control measures include complete destruction of diseased animals and animals being in contact with them, as well as imposing quarantine in the area within a radius of 10-kilometers around the established outbreak.

However, elimination of ASF outbreaks depends not only on biosafety level of pig farms and the effectiveness of the veterinary service, but also on the presence of transmission vectors and pathogen distribution factors. The high density of wild boar population in Europe and Southeast Asia does not allow for the rapid elimination of ASF virus in the wild without the use of specific prophylaxis agents, which have not yet been developed.

Conflict of interest. The authors declare that there is no conflict of interest.

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